

AD_____

Award Number: DAMD17-98-1-8114

TITLE: Ribozyme-Mediated Breast Cancer Gene Therapy

PRINCIPAL INVESTIGATOR: Paula Marie Pitha-Rowe, Ph.D.
Lesia Dropulic, M.D.
Boro Dropulic, Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205-2196

REPORT DATE: April 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021104 102

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	April 2002	Annual Summary (1 Oct 98 - 31 Mar 02)	
4. TITLE AND SUBTITLE Ribozyme-Mediated Breast Cancer Gene Therapy			5. FUNDING NUMBER DAMD17-98-1-8114
6. AUTHOR(S) Paula Marie Pitha-Rowe, Ph.D. Lesia Dropulic, M.D. Boro Dropulic, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Johns Hopkins University School of Medicine Baltimore, Maryland 21205-2196 E-Mail: parowe@jhmi.edu			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES Report contains color			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT <i>(Maximum 200 Words)</i> In our laboratory we have developed lentiviral vectors for use in anti-breast cancer gene therapy. Our specific goal for this project was to evaluate the feasibility of using lentiviral vectors that express anti-HER-2/neu antisense or ribozymes for the treatment of breast cancer. The advantage of lentiviral vectors over other vectors is that lentiviral vectors can transduce human cells with great efficiency. We have demonstrated that HIV-based lentiviral vectors can transduce a variety of human cell types with up to 99% efficiency, as measured by FACS analysis of GFP expression. Specifically, these vectors can transduce human breast cancer cells with up to 90 to 97% efficiency. HER-2 overexpressing breast cancer cell lines, transduced with a lentiviral vector that expresses an anti-HER-2 antisense sequence, exhibit slower growth in vitro compared to non-transduced cells. In addition, breast cancer tumors in nude mice, formed by injection of breast cancer cells transduced with the anti-HER-2 lentiviral vector, tended to grow more slowly than tumors formed by non-transduced breast cancer cells. Modifications and improvements of the current vector system can potentially generate a potent genetic therapy for breast cancer.			
14. SUBJECT TERMS breast cancer, lentiviral vectors, HER-2, ribozymes, antisense			15. NUMBER OF PAGES 13
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4-11
Key Research Accomplishments.....	12
Reportable Outcomes.....	12
Conclusions.....	12
References.....	13
Appendices.....	13

I. Introduction.

The overall goal of this project is to develop novel lentiviral vectors for cancer gene therapy. Our specific goal for this project was to evaluate the feasibility of using lentiviral vectors that express anti-HER-2/neu antisense sequences for the treatment of breast cancer. The advantage of lentiviral vectors over other vectors is that these vectors can transduce human cells with great efficiency. The goals for this project were as follows: (1) construct lentiviral vectors and demonstrate efficient transduction of human cells, particularly human breast cancer cells, (2) test the anti-Her2/neu antisense expressing vectors for their ability to inhibit Her2/neu expression in tumor cell lines in vitro, and (3) test the vectors for their ability to decrease growth of breast cancer tumors in small animal models.

II. Body.

Technical objective #1: Develop and produce lentiviral vectors expressing antisense sequences against HER-2.

Task 1: Constructed basic vector and helper constructs for the production of high titer HIV-based lentiviral vectors.

HIV-vector constructs based on the original vectors designed by Dropulic et al (Dropulic 1996) were optimized to increase their transduction efficiency. The salient features of the vector are shown below in figure 1.

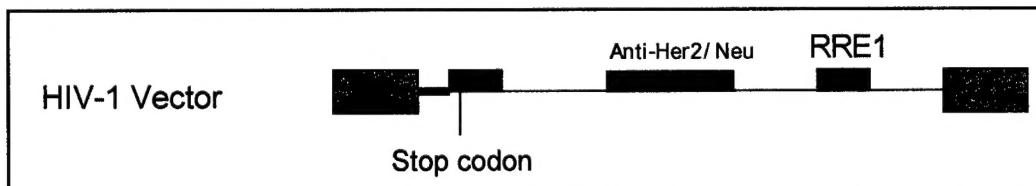


Figure 1. Salient features of HIV vectors. The antisense or ribozyme anti-Her2/neu payload (blue) is inserted upstream of the RRE (green) element that is flanked by two HIV-LTRs (grey). The packaging sequence (purple) contains a stop codon 41 nucleotides from the start of transcription to prevent translation of a Gag sequence that would be immunogenic since the first known CTL epitope for HIV gag is downstream of the stop-codon modified site. An optional green fluorescent protein (GFP) gene is present downstream of the RRE sequence.

New VIRPAC helper production system for production of high titer HIV-based vectors

A new helper production system was established for high efficiency HIV-based vector production. The production system uses a VIRPAC helper plasmid that codes for the HIV structural proteins and the VSV-G envelope protein. Therefore, HIV-based vectors have a broad tropism for many primary and tumorigenic cell

types, including breast cancer cells. Using NUNC cell factories, we can produce in excess of 10^{10} transducing vector units per cell factory.

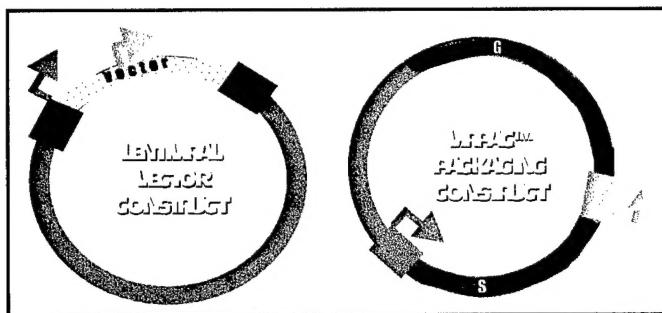


Figure 2. Schematic representation of the vector and the VIRPAC packaging constructs used for transient production of the HIV-vector in 293T cells. On the left, the vector construct shows two green LTRs with a yellow backbone. The red arrow shows the start of genomic vector RNA transcription while the blue arrow shows the start of the anti-HER2/neu antisense RNA transcription. On the right, the VIRPAC helper construct shows the S coding sequence in blue. This sequence codes for the HIV structural and enzymatic proteins while the purple G sequence codes for the VSV-G envelope protein that confers broad tropism to the HIV vector.

High efficiency transduction of a variety of primary human cell types and breast cancer cell lines.

Conditions for efficient transduction of a variety of primary human cells and tumor cell lines were optimized. We found that many primary human cells could be stably transduced with greater than 90% efficiency, as measured by GFP expression. For example, 92.8% of primary human CD4+ T cells was transduced with a GFP-expressing HIV vector, as determined by FACS analysis of GFP positive cells.

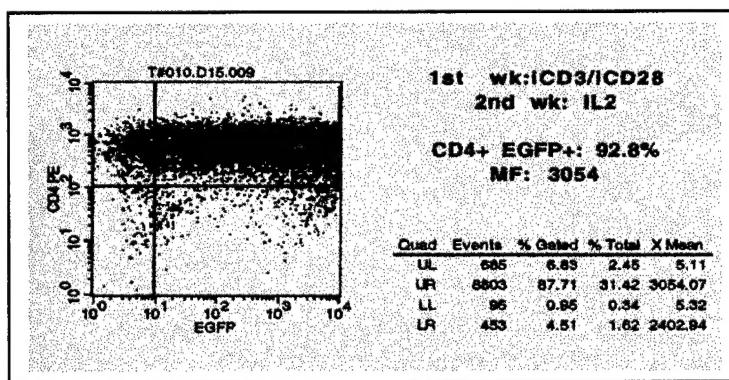


Figure 3. An example of high efficiency transduction of primary human cells by an HIV-based vector. CD4+ T cells were isolated from a donor and incubated with the vector at a multiplicity of infection of 20 in media containing iCD3/28 during the first week and IL-2 in the second week. Analysis by FACS revealed that over 90% of the cells were GFP

positive, demonstrating that at least 90% of the cells stably contained the vector. Significantly, no vector-related toxic effects were observed.

We have also worked on transduction methods for other primary human cell types. The CD34+ stem cell is an important cell type for human gene therapy. We are able to efficiently transduce CD34+ stem cells derived from human cord blood with the HIV-based lentiviral vector. Strikingly, transduction efficiencies of over 90% were detected eight weeks after transplantation of the transduced cells in NOD/SCID mice.

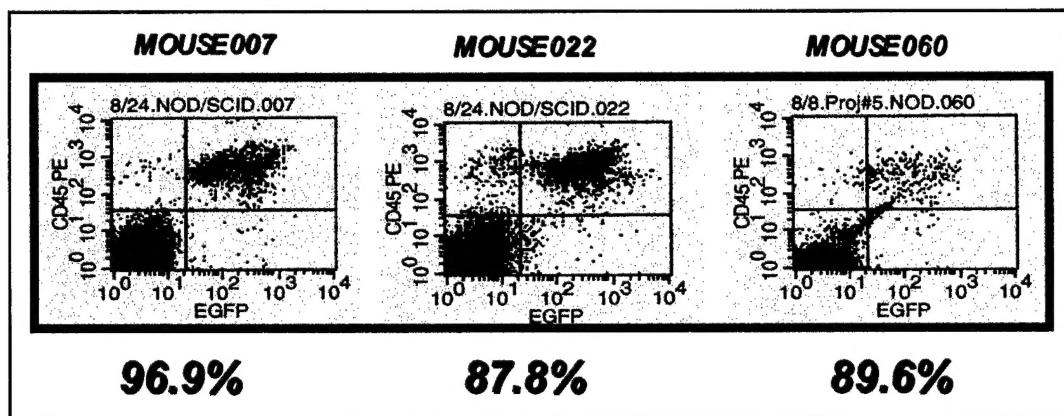


Figure 4. Efficient transduction of human CD34+ cells with an HIV-vector that expresses GFP. Cells were transduced with vector in the presence of a cytokine cocktail and then transplanted into NOD/SCID mice. Blood and bone marrow cells were harvested from the mice 8 weeks after the transplant and analyzed by FACS. The three animals shown above were one complete group. On average, over 90% of the human cells expressed GFP.

Conditions for efficient transduction of breast cancer cells were optimized. Varying multiplicities of infection (MOI) of HIV-based vectors were used to determine which MOI produced the highest level of transduction without adverse effects.

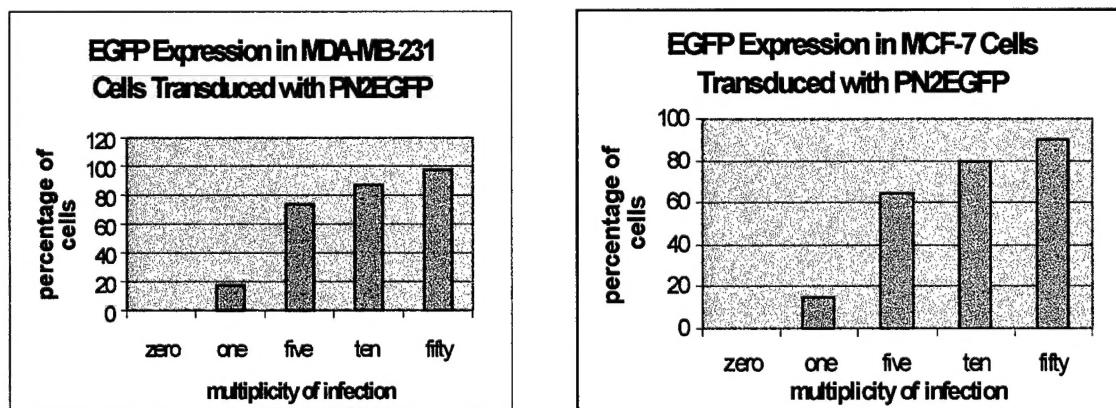


Figure 5. High efficiency transduction of the human breast cancer cell lines, MDA-MB-231 and MCF-7, by the HIV-based vector. The breast cancer cells were incubated with the lentiviral

vector, PN2EGFP, at varying MOIs and then analyzed by FACS analysis 2 days after transduction. Significantly, 90 to 97% transduction efficiency was achieved by using an MOI of 50 of the HIV-based lentiviral vector. Similar results were obtained with the HER-2-overexpressing breast cancer cell line, SKBR-3.

Technical objective #2: In vitro testing of anti-HER-2 lentiviral vectors in breast cancer cells.

Task 2: Examine the effects of the antisense sequence on HER-2 protein expression.

Protein immunoblotting was performed to determine whether the anti-HER2 antisense sequence expressed by the lentiviral vector resulted in decreased HER2 protein expression in breast cancer cell lines.

Various breast cancer cell lines were transduced with lentiviral vectors expressing either the anti-HER2 antisense sequence or the anti-MGMT (O^6 -methylguanine DNA-methyltransferase) antisense sequence, as a control. Seventy-two hours after transduction the cells were harvested by lysing with lysis buffer containing 150 mM NaCl, 10 mM EDTA, 1 % Triton-X 100, 0.5 % sodium deoxycholate, 50 mM Tris Cl [pH 8.0]. Fifteen micrograms of cell lysate were loaded onto a 10% SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane, the membranes were immunoblotted with anti-HER2 and anti-actin antibodies.

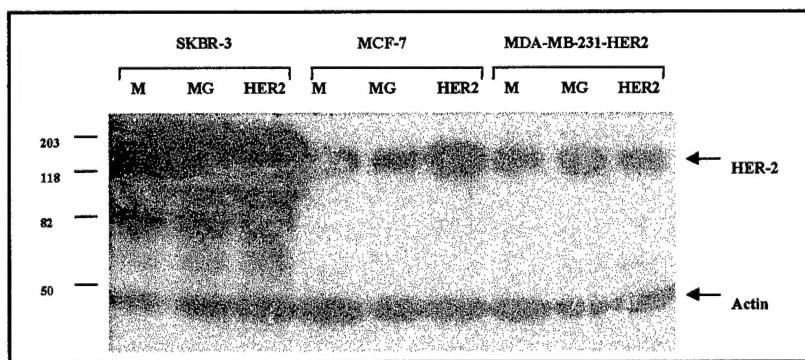


Figure 6. Immunoblot of SKBR-3, MCF-7, and MDA-MB-231-HER2 human breast cancer cell lines transduced with the anti-HER2 lentiviral vector, HER-2, or with the anti-MGMT lentiviral vector, MG. M = non-transduced cells.

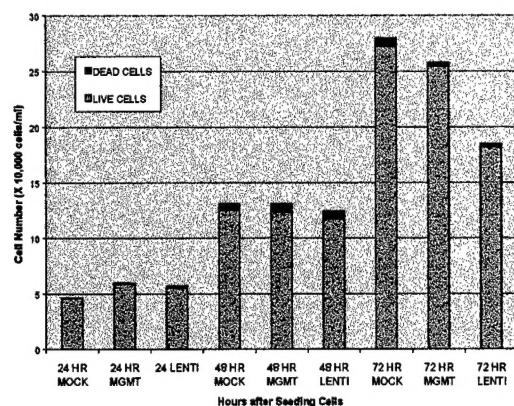
Conclusion: This experiment was repeated three times and no consistent decrease in the amount of HER-2 expression was observed in breast cancer cell lines transduced with the anti-HER2 vector compared to cell lines transduced with the anti-MGMT vector and to non-transduced cell lines. SKBR-3 cells express a large amount of HER-2. Even when less protein was loaded onto the gel and with shorter exposure times, no downregulation of HER-2 expression

was noted in anti-HER-2 lentivirus transduced cells. We expected to observe downregulation of HER-2 expression in cells transduced with the lentiviral vector expressing anti-HER-2 antisense. Further studies will be performed to determine whether the antisense effect can be observed at the RNA level.

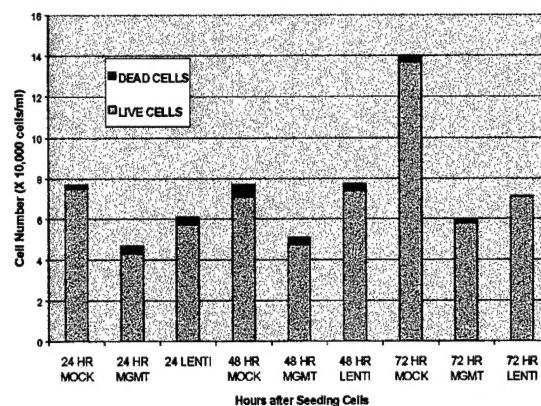
Task 3: Examine the effects of anti-HER-2 expression on the growth of human breast cancer cell lines in vitro.

To determine whether the lentiviral vector expressing antisense sequence against HER-2/Neu inhibited the growth of breast cancer cells, breast cancer cells that had been transduced with the anti-HER-2 vector, cells transduced with the anti-MGMT vector, or non-transduced cells were seeded in 24 well plates at 5×10^4 cells/well. At 24, 48, or 72 hours after seeding, the cells were harvested with Versene/trypsin (1:1), and the number of alive and dead cells was determined using the trypan blue exclusion assay.

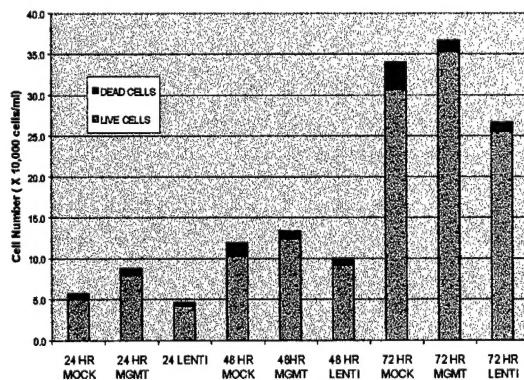
A. MDA-MB-231- HER2



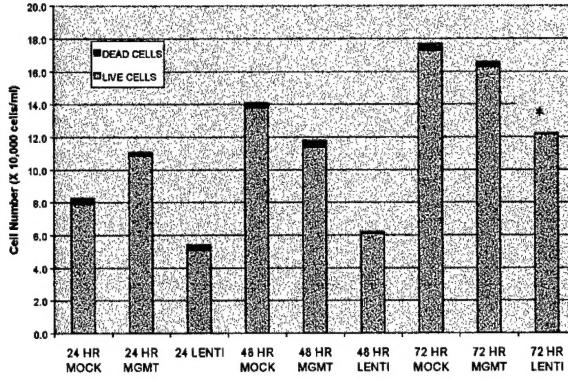
B. SKBR-3



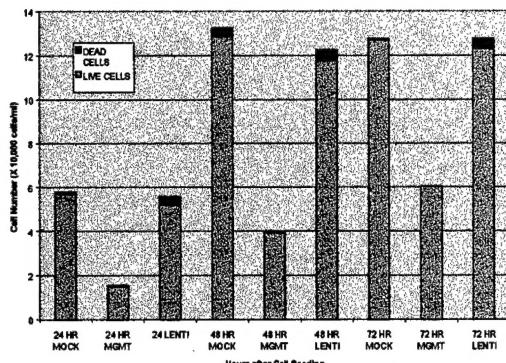
C. MDA-MB-231-HER2



D. SKBR-3



E. MCF-7



F. BHK-21

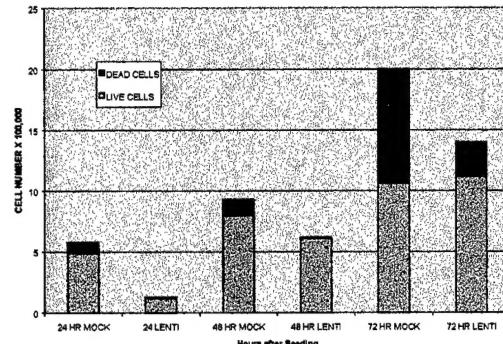


Figure 7. Growth of lentivirus vector transduced breast cancer cell lines. Graphs A and B are from experiment number 1 in which each sample was run in duplicate and the average number of live and dead cells is graphed. Graphs C and D are from experiment number 2 in which each sample was run in triplicate and the average number of live and dead cells is graphed. Graphs A – D are of cells that overexpress HER-2, MDA-MB-231-HER2 and SKBR-3 cells. Graphs E and F are of cells that express lower levels of HER-2, MCF-7 and BHK-21 cells, respectively. Mock = non-transduced; MGMT= transduced with the lentiviral vector expressing anti-MGMT antisense sequence; LENTI= transduced with lentiviral vector expressing anti-HER-2 antisense sequence. The asterisk denotes statistically significant data compared to the mock sample as determined by the Student's t test.

Conclusions: Breast cancer cell lines that express high levels of HER-2 and that are transduced with the anti-HER-2 lentiviral vector exhibited a trend to grow more slowly compared to mock transfected cell lines. Whereas, cells that do not overexpress HER-2, MCF-7 and BHK-21 cells, did not exhibit this trend. These data suggest that the antisense sequence against HER-2 expressed by the lentiviral vector inhibits the growth of HER-2 overexpressing cell lines. Soft agar assays will be performed to assess the clonogenic capacity of the transduced breast cancer cell lines.

Technical objective #3: In vivo testing of the anti-HER-2 lentiviral vector in nude mice.

Task 4: Characterization of the ability of anti-HER-2 lentiviral vectors to inhibit tumor growth in vivo.

We determined whether the lentiviral vector that expresses the antisense sequence against HER-2 can inhibit tumor formation and growth in vivo. Two million MDA-MB-231 human breast cancer cells transduced with the lentiviral vector expressing anti-HER-2 antisense sequence or the anti-MGMT antisense sequence or non-transduced cells were injected bilaterally into the mammary fat pads of 6-week-old nude mice (figure 6). In addition, MDA-MB-231 cells stably

overexpressing HER-2 (MDA-MB-HER2) and transduced or not transduced with the lentiviral vectors were injected into the mammary fat pads of nude mice (figure 7). After injection, the mice were observed for tumor formation and tumor growth for 26 to 57 days. Six mice were used for each cell line and each vector. Tumor length and width was recorded every two days using calipers and the tumor volume was calculated using the following formula: length \times width \times 0.5 (Clinchy *et al.*). A mouse was euthanized when a tumor diameter of 1.5 cm was reached or when the mouse exhibited difficulty walking, difficulty breathing, developed ulcers in the skin of tumor bearing regions, or exhibited a significant decrease in overall activity level.

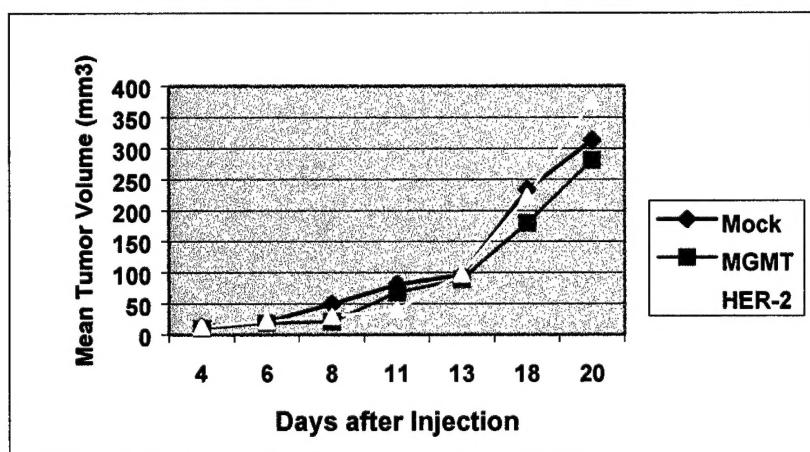


Figure 8. Mean tumor volume after injection of nude mice with MDA-MB-231 cells transduced with lentiviral vectors. Mock = non-transduced cells; MGMT = lentiviral vector contains anti-MGMT antisense; HER-2 = lentiviral vector contains anti-HER-2 antisense.

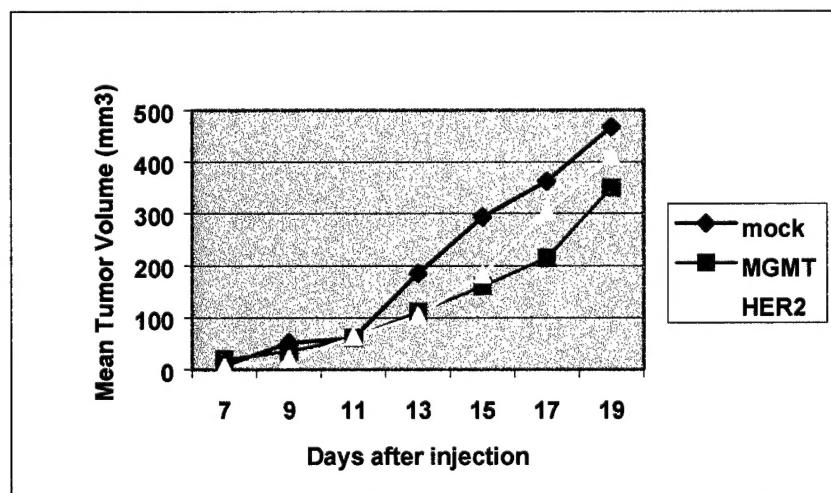


Figure 9. Mean tumor volume after injection of MDA-MD-231-HER2 cells transduced with lentiviral vectors. Mock = not transduced; MGMT = lentiviral vector expresses anti-MGMT antisense; HER2 = lentiviral vector expresses anti-HER-2 antisense sequence.

Conclusions: The tumors formed after injection of mice with MDA-MB-231 cells that were transduced or not transduced with the lentiviral vectors exhibited a similar rate of growth. However, the tumors formed by injection of MDA-MB-231-HER-2 cells that were transduced with the anti-HER-2 or anti-MGMT antisense expressing vectors tended to grow slower than tumors arising from non-transduced cells. These data were not statistically different as determined by the Student's t-test. However, these data suggest that the lentiviral vectors expressing the anti-HER2 antisense or the anti-MGMT antisense sequences have an effect on tumor growth in a HER-2 overexpressing breast cancer cell line. Most mice were sacrificed by day 27 because the tumors had reached a maximum size of 1.5 cm in diameter. However, two mice injected with MDA-MB-231 cells that were transduced with the anti-HER-2 lentiviral vector had bilateral tumors that were very slow growing and did not reach the maximum allowed size until day 57. These data further suggest that a lentiviral vector expressing an antisense sequence against an oncogene can inhibit the growth of the tumor.

III. Key Research Accomplishments.

- Development of a vector and helper system for high efficiency production of HIV vectors.
- Achieved high efficiency transduction of primary human cells and several breast cancer cell lines.
- Tested the lentiviral vector construct expressing the anti-HER2 antisense for the ability to downregulate HER-2 expression.
- Determined whether expression of the anti-HER2 antisense in breast cancer cell lines has an effect on growth of cancer cells in vitro.
- Determined whether the expression of the anti-HER2 antisense in breast cancer cells has an effect on tumor formation and tumor growth in vivo.

IV. Reportable Outcomes.

- Either the Johns Hopkins University or Virxsys Corporation have submitted all intellectual property pertaining to the vector system to the USPTO. No new intellectual property was developed during the course of the work.
- Various breast cancer cell lines stably expressing the lentiviral vector constructs expressing the anti-HER-2 or anti-MGMT antisense or the enhanced green fluorescent protein have been developed.
- Ms. Holly Hammond, a research technician who worked on this project, is now a research technician in the Johns Hopkins Cancer Center. Based on her experience gained from work on this project, she is working in a similar area of breast cancer research.

V. Conclusions. We have developed a powerful vector system for efficient transduction of human breast cancer cells. We have developed anti-HER2/neu antisense HIV-vectors that we tested in breast cancer cells for (1) their ability to down-regulate HER2/neu expression in vitro and (2) their ability to decrease the tumorigenicity of HER-2-expressing breast cancer cells in small animal models. We noted a trend towards decreased breast cancer cell growth in vitro and decreased size of breast cancer tumors in small animal models. Improvements in the current vector system are anticipated to enhance these effects. This vector system has the potential to be used with other promising anti-breast cancer genes to produce a potentially potent genetic therapy for breast cancer that may be used independently or in conjunction with other therapeutic modalities .

VI. References.

- Dropulic B, Hermankova M, Pitha PM.** A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread. Proc Natl Acad Sci USA 1996 ;93: 11103-11108.
- Clinchy B, Gazdar A, Rabinovsky R, Yefenof E, Gordon B, Vitetta ES.** The growth and metastasis of human, HER-2/neu-overexpressing tumor cell lines in male SCID mice 2000; 61: 217-228.

VII. Appendix.

Personnel receiving pay from this research effort:

Lesia K. Dropulic, M.D.
Holly H. Hammond.